Molecular Changes in Second Primary Lung and Breast Cancers after Therapy for Hodgkin's Disease[1]

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Abstract

The risk of lung and breast cancer is significantly increased after therapy for Hodgkin's disease (HD), but there are few data that describe the molecular profiles of these tumors. We investigated the genetic abnormalities in second primary lung ($n = 19$) and breast cancers ($n = 19$) that follow therapy for HD ("post-HD cancers") and compared these with changes observed in corresponding tumor types (57 lung and 20 breast cancers) arising in the general population ("sporadic cancers"). DNA obtained from archival tissues was examined using PCR-based analyses for loss of heterozygosity and microsatellite alterations (MAs) at several chromosomal regions, *TP53* and K-*ras* gene mutations, and frameshift mutations at minisatellite sequences at the coding regions of several genes (*TGF-βRII*, *IGFIIR*, *BAX*, *hMSH6*, and *hMSH3*). The occurrence of loss of heterozygosity at all chromosomal regions taken together and frequencies at most individual areas were similar for the post-HD and sporadic cancers for both lung and breast sites. The overall frequency of MAs in the post-HD tumors was substantially greater (lung, 2.4-fold, $P = 0.004$; breast, 4.2-fold, $P = 0.16$) than that in the respective sporadic cancers. No differences in the pattern of *TP53* and K-*ras* mutations were detected between post-HD and sporadic cancers. No mutations were detected at the minisatellite sequences examined. MAs, which reflect widespread genomic instability, occur at greatly increased frequency in post-HD lung and breast cancers. Although the mechanisms underlying the development of increased MAs are unknown, they have been associated with immunosuppression and radiation exposure. Future research should address the role that MAs, as well as other influences, may play in the development of neoplasias that occur after therapy for HD.

Introduction

The development of effective radiotherapy and chemotherapy regimens for the treatment of HD[3] has resulted in large numbers of long-term survivors. Second primary malignancies are a serious complication of cancer therapy for these patients, who demonstrate a 15–20-year actuarial risk of approximately 20% (1, 2). In particular, significant excesses of lung and breast cancer have been observed among HD survivors; lung cancer occurs at a rate 2–8 times that seen in the general population (1–5), with a portion of this increase related to thoracic radiotherapy (6, 7) and possibly to chemotherapy (6). An increased risk of breast cancer has been observed among women treated for HD before age 30 years (8–11), with many cases following mantle radiotherapy that includes exposure to the breast.

However, there are few data on the molecular changes that accompany lung (12) or breast cancer after treatment for HD; this type of data might provide further insight into the contribution of various etiological factors and carcinogenic pathways compared with *de novo* tumors. For clinically evident sporadic lung cancers, multiple genetic changes (estimated to be at least 10–20) have been described in known TSGs and several dominant oncogenes, including *myc* family members, K-*ras*, *TP53*, *RB*, *p16-CDKN2*, and candidate TSG regions on chromosome 3p, 5q22 (*APC-MCC* region), 8p, 11p and others (13). Breast cancer pathogenesis is characterized by multiple molecular changes, including activation of oncogenes and loss of known and putative TSGs, including HER2/neu, *TP53*, and candidate TSG regions on chromosomes 3p, 6q, 8p, 11p, 13q, 16q, 17p, 17q, and 18q (14–16). Microsatellites, which are short tandem repeat DNA sequences, are abundantly and evenly distributed throughout the genome. Because they are easily analyzed by PCR-based methods (17), they are frequently used for studies of LOH in tumors, including those of the breast and lung. The fidelity of normal microsatellite replication appears to be relatively low because these loci are highly polymorphic (18), presumably due to unrepaired slippage during DNA replication (19). In addition to LOH, changes in microsatellite size (both additions and deletions) are associated with many cancers and other human diseases. These alterations have been linked with

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[3] The abbreviations used are: HD, Hodgkin's disease; LOH, loss of heterozygosity; MA, microsatellite alteration; TSG, tumor suppressor genes; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; FAL, fractional allelic loss; FRL, fractional regional loss.

at least four disease states: (*a*) hereditary nonpolyposis colon cancer, in which inherited defects in DNA mismatch repair enzymes result in large-scale genetic instability with the formation of a ladder-like array of microsatellites of various sizes replacing the normal allele pattern (18, 20); (*b*) another form of microsatellite change in which only a single allele of altered size is found has been described in many forms of sporadic cancers (21–23), including lung cancer, and is referred to as MA (MAs usually involve dinucleotide repeats and occasionally involve the less common multinucleotide repeats); (*c*) in the Ashkenazi Jewish population, a T to A polymorphism at *APC* gene nucleotide 3920 results in a hypermutable tract of $(A)_8$ sequences, indirectly causing a predisposition to familial colorectal cancer (24); and (*d*) expansion of unstable trinucleotide repeats in the coding regions of some genes results in a number of familial neuromuscular disorders (25).

In the present report, we investigated the frequency and pattern of *TP53* and K-*ras* gene mutations and the occurrence of LOH and MAs at several chromosomal regions frequently deleted in lung and breast tumors in a series of 38 cases that followed therapy for HD. Results are compared with findings in sporadic tumors of the lung and breast in the general population.

Materials and Methods

Lung and Breast Tumor Specimens. Paraffin-embedded archival tissues were collected as available for all HD patients who developed a second lung or breast cancer and were reported to cancer registries [Iowa and Connecticut (United States) and Ontario (Canada)] included in an ongoing epidemiological study. Calendar years of HD diagnosis were 1965–1990, and eligible second cancers occurred 1 year or more later. Specimens were analyzed for 38 HD patients with either second lung ($n = 19$) or breast ($n = 19$) cancer (post-HD cancers). The lung tumors included 15 NSCLCs (10 adenocarcinomas, 1 adenosquamous carcinoma, 1 squamous cell carcinoma, and 3 large cell carcinomas) and 4 SCLCs (Table 1). Patients (8 females and 11 males) ranged in age from 20–61 years at diagnosis of HD (median age at diagnosis, 37 years). The median latency between diagnosis of HD and lung cancer was 10.1 years (range, 2.3–24.6 years). Radiotherapy for HD was administered to all patients, with 13 patients also receiving chemotherapy. All patients were smokers, and 13 of 14 patients for whom quantitative data were available were heavy smokers (more than 20 pack-years), with a median of 37.5 pack-years (range, 13–79.5 pack-years). Findings from the post-HD lung cancers were compared with archival materials from 57 lung tumors arising in the general population (sporadic cancers) from patients (21 males and 26 females) undergoing curative intent resections, with all major histological types represented. These sporadic lung cancers, which were obtained from Parkland Hospital (Dallas, TX) and The University of Texas M. D. Anderson Cancer Center (Houston, TX), consisted of 35 NSCLCs (21 adenocarcinomas and 14 squamous cell carcinomas) and 22 SCLCs. The NSCLC samples were selected randomly from the large series available to us; because SCLC tumors are seldom resected, even when apparently limited in extent, we analyzed all available resected cases. Given the rarity of sporadic lung cancer in the young, the median age of the comparison patients was considerably high (median age, 61 years, range, 30–84 years) as compared with age at lung cancer diagnosis (median age, 51 years, age range, 28–67 years) after HD. Eight of the patients with sporadic lung cancer were <50 years old, and all but one were heavy smokers, with a median smoking exposure of 37.5 pack-years.

All post-HD breast cancers were ductal cell carcinomas obtained from patients who had been treated for HD at 30 years of age or younger (median age, 21 years, age range, 13–28 years; Table 1). The median latency between diagnosis of HD and breast cancer was 16.3 years (range, 9.3–26.4 years). Breast cancer was diagnosed at a median age of only 37 years (range, 28–53 years). Eighteen patients had received chest radiotherapy for HD. Molecular findings of these cases were compared with those derived from 20 ductal breast carcinomas arising among women in the general population who underwent curative intent mastectomy (women with sporadic cancers). The patients with sporadic breast cancer ranged in age from 33–66 years (median age, 50 years).

Microdissection and DNA Extraction. Precisely identified areas of lung and breast cancers were microdissected under microscopic visualization using laser capture microdissection (LCM; Arcturus Engineering, Mountain View, CA) without contamination with normal stromal cells. Stromal cells or lymphocytes from the same sections were used as a source of constitutional DNA. After DNA extraction, 5 μl of the proteinase K-digested samples containing DNA from at least 100 cells were used for each multiplex PCR reaction using methods described previously (26).

***TP53* and K-*ras* Gene Mutation Analyses.** We examined post-HD lung and breast cancers for mutations in exons 5–8 of the *TP53* gene by single-strand conformational polymorphism analysis followed by sequencing of both strands of abnormal bands using an automated ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Branchburg, NJ). The nested PCR methodology, single-strand conformational polymorphism analysis, and primer sequences used have been described previously (22). For K-*ras* mutation analysis (codons 12 and 13) of post-HD lung cancers, we used a designed RFLP method using nested PCR methodology followed by sequencing, as described previously (27). K-*ras* gene mutations thus discovered were confirmed by direct sequencing using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer).

Polymorphic DNA Markers and PCR for LOH and MA Analyses. To evaluate LOH and MA in lung cancer, we used primers flanking dinucleotide ($n = 15$) and multinucleotide ($n = 5$) microsatellite repeat polymorphisms located at the following nine chromosomal regions: (*a*) 3p12 (*D3S1274* and *D3S1284*); (*b*) 3p14.2 (*D3S1234* and *D3S4103* at the *FHIT* gene); (*c*) 3p14–21 (*D3S1766*); (*d*) 3p21 (*D3S1076*, *D3S1573*, *D3S1029*, *D3SS1582*, *ITIH-1*, and *Luca 2.2*); (*e*) 3p22–24.2 (*D3S1612*, *D3S2432*, and *D3S1537*); (*f*) 5q21 (*APC-MCC* region, L5.71); (*g*) 9p21 (*IFNA* and *D9S1748* flanking the *CDKN2* gene); (*h*) 13q14 (*RB* gene, dinucleotide repeat); and (*i*) 17p13 (*TP53* gene, dinucelotide and pentanucleotide repeats). For breast cancer, we used primers flanking dinucleotide ($n = 16$) and multinucleotide ($n = 4$) microsatellite repeat polymorphisms located at the following 13 chromosomal regions: (*a*) 3p14.2 (*D3S1234* and *D3S4103* at the *FHIT* gene); (*b*) 3p14–21 (*D3S1766*); (*c*) 3p21 (*ITIH-1* and *Luca 2.2*); (*d*) 3p22–24.2 (*D3S2432*); (*e*) 5q21 (*APC-MCC* region, L5.71); (*f*) 6q13–14 (*D6S300*); (*g*) 6q22–27 (*D6S262*); (*h*) 8p21–23 (*D8S1130*, *D8S1106*, and *NEFL*); (*i*) 9p21 (*IFNA*, flanking the *CDKN2* gene); (*j*) 11q13 (*PYGM* and *INT-2*); (*k*) 13q14 (*RB* gene, dinucleotide repeat); (*l*) 17p13 (*TP53* gene, dinucelotide and pentanucleotide repeats); and (*m*) 17q21 (*D17S855* and *D17S1323* flanking the *BRCA1* gene). Primer sequences were obtained from the Genome Database, with five exceptions

Table 1 Clinicopathological and molecular data for patients with lung cancer or breast cancer after HD

A. Lung cancer

Patient no.	Histological type of LC[a]	Age[b]/ sex	Smoking history[c]	Cancer location	Years between HD/LC	HD stage	Summary of therapy for HD	FAL index	MA index
1	Adenocarcinoma	44/F	Yes	RUL	2.3	II	Mantle RT 30 Gy; MOPP-ABVD	0.69	0.21
2	Adenocarcinoma	37/M	22	LUL	10.2	IV	Mantle RT 21 Gy; Abd. RT 18 Gy; MVVPP	0.07	0
3	Adenocarcinoma	31/F	40	RLL	16.1	II	Mantle RT 46 Gy	0	0
4	Adenocarcinoma	61/M	79.5	LLL	6.3	I	Mantle RT 35 Gy; MOPP	0.08	0.06
5	Adenocarcinoma	32/M	Yes	RL	10.1	I	Hemi-mantle RT 44 Gy	0.50	0.05
6	Adenocarcinoma	34/M	27	LLL	21.8	I	Mantle RT 18 Gy; inverted-Y RT 36 Gy; CTX, VLB	0.46	0.16
7	Adenocarcinoma	59/M	70	RML	2.5	I	Inverted-Y RT 35 Gy	0.45	0.11
8	Adenocarcinoma	34/F	30	RML	3.9	II	Mantle RT 30 Gy; MOPP	0.56	0.11
9	Adenocarcinoma	28/M	35	LUL	23.3	II	Inverted-Y RT 34 Gy; MOPP	0.67	0.37
10	Adenocarcinoma	40/F	60	LUL	7.8	I	Lateral neck RT 35 Gy	0.20	0.05
11	Adeno-squamous	59/M	50	RUL	3.0	II	Lateral pelvis RT 25 Gy; MTX; ABVD	0.91	0.11
12	Squamous cell	40/F	40	LL	24.6	IV	Lateral Axilla RT 36 Gy; MOPP	0.67	0.12
13	Large cell	58/M	Yes	RL	9.8	I	Mantle RT 40 Gy; para-aortic RT 40 Gy; MOPP	0.83	0
14	Large cell	26/M	30	LUL	22.0	I	Mantle RT 16 Gy; Inverted-Y RT 36 Gy; MOPP	0.70	0
15	Large cell	34/F	Yes	RML	22.1	II	Mantle RT 30 Gy; CVP	0.44	0.16
16	SCLC	56/M	Yes	RUL	10.9	II	Mantle RT 40 Gy	0.60	0
17	SCLC	32/F	28	Unknown	15.0	II	Mantle RT 35 Gy	1.00	0.05
18	SCLC	49/F	40	LUL	6.1	II	Mantle RT 40 Gy; MOPP; ABVD	0.73	0.05
19	SCLC	20/M	13	LUL	8.8	I	Mantle RT 35 Gy; MOPP; −ABVD	0.17	0.05

B. Breast cancer

Patient no.	Histological type of BC	Age[b]/ sex	BC location	Years between HD/BC	HD stage	Summary of therapy for HD	FAL index	MA index
1	Ductal cell	41/F	R, lateral	18.3	III	Mantle RT 30 Gy; Abd/pelvis RT 23 Gy; MOPP	0.85	0.15
2	Ductal cell	43/F	R and L, LOQ-LIQ	16.3	II	Mantle RT 35 Gy; Abd RT 20 Gy; para-aortic RT 10 Gy; MOPP	0.20	0
3	Ductal cell	35/F	R, UOQ	16.3	II	Mantle RT 35 Gy	0.40	0.05
4	Ductal cell	32/F	R, central	19.0	II	Mantle RT 35 Gy; right chest RT 10 Gy; Mediast. RT 20 Gy; VLB	0.69	0
5	Ductal cell	40/F	R, UOQ	14.3	IV	MOPP	0.85	0
6	Ductal cell	35/F	L, LIQ	9.8	II	Mantle RT 35 Gy; left chest RT 20 Gy; MOPP	0.17	0
7	Ductal cell	42/F	L, UOQ	21.1	II	Mantle RT 35 Gy; left chest RT 3 Gy; VLB	0.13	0.10
8	Ductal cell	38/F	L, upper left	19.8	II	Mantle RT 40 Gy	0.67	0
9	Ductal cell	34/F	L, UIQ	13.2	II	Mantle RT 38 Gy; para-aortic RT 38 Gy; MVPP	0.33	0.05
10	Ductal cell	43/F	L, LOQ	21.7	I	Mantle RT 40 Gy; MOPP	0.24	0
11	Ductal cell	53/F	L, UOQ	26.4	III	Mediast. RT 30 Gy; supracl left axilla RT 40 Gy	0.25	0.05
12	Ductal cell	30/F	L, UOQ	9.3	II	Mantle RT 35 Gy; Abd RT 20 Gy; para-aortic RT 15 Gy	0.27	0
13	Ductal cell	36/F	L, LOQ	21.8	II	Mantle RT 35 Gy	0.69	0
14	Ductal cell	52/F	L, UIQ	25.1	I	Mantle RT 26 Gy; Mediast. RT 8 Gy; axilla RT 4 Gy; left chest 40 Gy	0	0
15	Ductal cell	29/F	R, LIQ	13.7	I	Mantle RT 40 Gy; MVPP	0.14	0
16	Ductal cell	43/F	L, UOQ	15.8	II	Mantle RT 35 Gy; Abd. RT 4 Gy	0.18	0
17	Ductal cell	29/F	L, inferior central	14.2	II	Mantle RT 40 Gy; para-aortic/spleen RT 41 Gy	0.38	0
18	Ductal cell	40/F	L, UOQ	12.3	II	Mantle RT 38 Gy; MOPP	0.62	0
19	Ductal cell	34/F	L, UOQ	19.9	III	Mantle RT 46 Gy; inverted-Y/spleen RT 45 Gy	0.20	0

[a] LC, lung cancer; Abd, abdomen; ABVD, doxorubicin, bleomycin, vinblastine, and dacarbazine; BC, breast cancer; CTX, cyclophosphamide; CVP, cyclophosphamide, vincristine, and prednisone; L, left; LL, left lung; LLL, left lower lobe; LOQ, left outer quadrant; LIQ, left inner quadrant; LUL, left upper lobe; Mediast., mediastinal; MOPP, mechlorethamine, vincristine, procarbazine, and prednisone; MTX, methotrexate; MVPP, mechlorethamine, vinblastine, procarbazine, and prednisone; MVVPP, mechlorethamine, vincristine, vinblastine, procarbazine, and prednisone; R, right; RL, right lung; RLL, right lower lobe; RLL, right middle lobe; RT, radiotherapy; RUL, right upper lobe; supraclav, supraclavicular; UIQ, upper inner quadrant; UOQ, upper outer quadrant; VLB, vinblastine.
[b] Age (years) at diagnosis of second primary cancer.
[c] Smoking history is given in pack-year. Yes, smoker, but the number of pack-years is unknown.

(*ITIH-1*, pentanucleotide and dinucleotide repeats in the *TP*53 gene, and dinucleotide repeats in the *RB* gene and *Luca 2.2*), which were published and referenced previously (26). Nested PCR or two-round PCR (using the same set of primers in two consecutive amplifications) methods were used as described previously (26). Multiplex PCR was performed during the first amplification, followed by uniplex PCR for individual markers.

For each case, constitutional heterozygosity was determined by examination of normal stromal tissue. LOH was scored by visual detection of the complete absence of one tumor allele in heterozygous (*i.e.*, informative) cases (Fig. 1). MAs were detected by a shift in the mobility of one allele (Fig. 1), irrespective of whether or not it was accompanied by LOH.

Minisatellite Mutation Analyses at the *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH6*, and *hMSH3* Genes. Because we noted a higher frequency of MAs in post-HD lung carcinomas, we examined five minisatellite sequences in the coding regions of the *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3*, and *hMSH6* genes for the presence of frameshift mutations. These genes have been re-

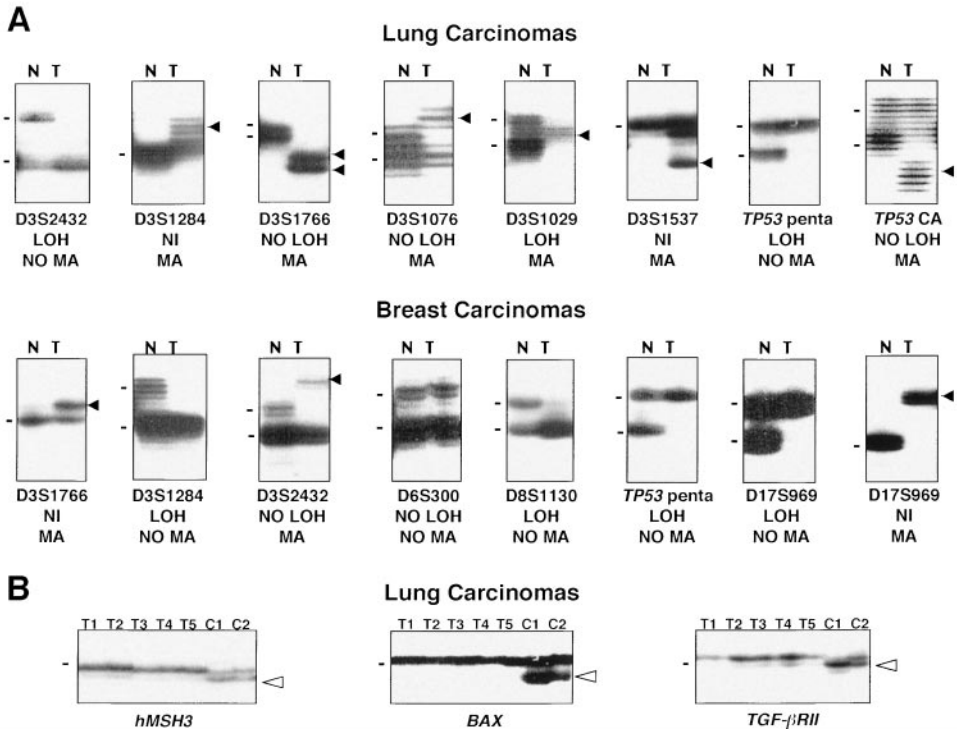

Fig. 1. *A*, 16 representative examples of LOH and MA analyses at eight chromosomal regions frequently deleted in post-HD lung and breast cancers. Each panel demonstrates microsatellite marker analysis of microdissected DNA of paired normal (*N*) and lung tumor (*T*) samples from individual patients. *Bars* represent the position of the major allelic bands. *Arrowheads* indicate a mobility shift of one or both alleles. *B*, representative examples of minisatellite mutation analysis at *hMSH3*, *BAX*, and *TGF-βRII* genes in post-HD lung carcinomas (*T1—T5*). *C1* and *C2*, colon cancer cell lines used as positive controls with frameshift mutations at those minisatellites sequences. *Open arrowheads* indicate a mobility shift (mutation) in the corresponding minisatellite sequences.

ported as mutational targets in tumors arising in patients with inherited defects in mismatch repair enzymes (28, 29). The minisatellite repeat sequences examined were as follows: (*a*) tracts of $(A)_{10}$ sequence at *TGF-βRII*; (*b*) $(G)_8$ at *IGFIIR*; (*c*) $(G)_8$ at *BAX*; (*d*) $(A)_8$ at *hMSH3*; and (*e*) $(C)_8$ at *hMSH6*, which were examined using the primer sequences published previously (29). A two-round PCR method for DNA extracted from paraffin-embedded tissues was used as described previously (26). Multiplex PCR was performed during the first amplification, followed by uniplex PCR for individual markers. Colon cancer cell lines having known frameshift mutations at those minisatellite sequences were used as positive controls. DNA from these cell lines was a gift from Dr. Jae-Gahb Park (Cancer Research Institute, Seoul National University College, Seoul, Korea).

Data Analysis. To compare the overall frequencies of LOH and MA in lung and breast carcinoma cases, we devised three indices (30), which were calculated calculated as follows: (*a*) FAL index = total number of loci with LOH/total number of informative loci; (b) FRL index = total number of chromosomal regions with LOH/total number of informative regions; and (*c*) MA index = total number of loci demonstrating MA/total number of loci analyzed.

The FAL index indicates the overall frequency of LOH at informative loci per case (maximum of 20 loci/case). The FRL index indicates LOH for all informative chromosomal regions per case (maximum of 9 and 13 regions/case for lung and breast cancers, respectively). In some instances, we were able to increase the number of regions that were informative by using multiple markers to analyze individual regions. If a marker for a region was informative (*i.e.*, heterozygous in normal tissue), then the region was regarded as informative, and if one or more of the markers showed LOH, then we regarded the region as demonstrating loss. The MA index indicates the total frequency of MAs expressed as a fraction per case ($n = 20$ markers tested in each type of tumor). Because MAs at individual markers occur independently of chromosomal region and informativeness, data from all markers were used. Because of the relatively large number of post-HD NSCLCs ($n = 15$), including 10 lung adenocarcinomas, we also included them in subgroup evaluations.

Statistical comparisons were performed using the exact Wilcoxon's rank-sum test and Fisher's exact test. Two-sided *P*s are reported for all comparisons.

Results

***TP53* and K-*ras* Mutations.** *TP53* gene mutations were detected in 11 of 19 (58%) lung carcinomas in patients treated for HD (Table 2). Whereas 3 of 4 (75%) SCLCs demonstrated *TP53* mutations, *TP53* mutations were detected in 8 of 15 (53%) NSCLCs [4 of 10 adenocarcinomas, 0 of 1 adenosquamous carcinoma, 1 of 1 squamous cell carcinoma, and 3 of 3 large cell carcinomas (one at each codon)]. Seven of the 10 (70%) *TP53* point mutations were transversions. K-*ras* gene mutations at codons 12 and 13 were detected in only 2 of 19 (11%) post-HD lung cancers, and both of these cases were adenocarcinomas. The mutation at codon 12 was a GGT (Gly) to TGT (Cys), and the mutation at codon 13 was a GAC (Asp) to GGC (Gly). *TP53* mutations were found in 2 of 19 (11%)

Table 2 *TP53* mutations in post-HD lung and breast tumors

Tumor type	Case no.	Exon/codon	Base substitution/amino acid change	Type of mutation
Lung	2	Exon 7/codon 245	1-bp insertion (T)	Frameshift
Lung	3	Exon 5/codon 135	TGC to TGG/Cys to Trp	C to G, transversion
Lung	8	Exon 5/codon 141	TGC to TGG/Cys to Trp	C to G, transversion
Lung	10	Exon 5/codon 179	CAT to CGT/His to Arg	A to G, transition
Lung	12	Exon 7/codon 248	CGG to CTG/Arg to Leu	G to T, transversion
Lung	13	Exon 5/codon 154	GGC to GTC/Gly to Val	G to T, transversion
Lung	14	Exon 5/codon 164	AAG to AAC/Lys to Asn	G to C, transversion
Lung	15	Exon 5/codon 135	TGC to TGG/Cys to Trp	C to G, transversion
Lung	16	Exon 8/codon 275	TGT to TAT/Cys to Tyr	G to A, transition
Lung	18	Exon 7/codon 238	TGT to TGG/Cys to Trp	T to G, transversion
Lung	19	Exon 8/codon 283	CGG to TGG/Arg to Trp	C to T, transition
Breast	8	Exon 6/codon 213	CGA to CAA/Arg to Gln	G to A, transition
Breast	10	Exon 7/codon 248	CGG to CAG/Arg to Gln	G to A, transition

post-HD breast carcinomas. Both point mutations represented transitions.

Frequency of LOH. Frequencies of LOH and the patterns of individual allelic losses were comparable for the post-HD and sporadic lung cancers (Fig. 2*A* and Tables 3 and 4). Similar frequencies of LOH at all analyzed chromosomal regions were also observed for both groups, as represented by FAL and FRL indices. However, for post-HD breast cancers, significantly greater allelic losses were observed for 6q13–14, 9p21, and any 6q and 17p (*TP53* gene loci) as compared with sporadic cancers. Post-HD breast cancers also displayed slightly higher mean FAL and FRL indices (0.382 and 0.429, respectively) than sporadic cancers (0.248 and 0.299; $P = 0.12$ and 0.17, respectively).

In both post-HD lung and breast tumors, no correlation was detected between the overall frequency of LOH and the age at which HD treatment was received, sex, HD stage, chest radiotherapy or chemotherapy, or period of time between therapy and second cancer diagnosis. For both sporadic and post-HD lung cancers, no correlation between smoking exposure (pack-years) and the occurrence of LOH was evident.

Frequency of MAs. Although there was considerable interindividual variability (Table 1), the mean MA index of the post-HD lung tumors (0.087) was 2.4-fold greater than the mean index of the sporadic cases (0.036; $P = 0.004$; Fig. 2B). Larger differences between the two groups of lung carcinomas were detected in the NSCLC and adenocarcinoma cases considered separately. The mean MA index of the post-HD NSCLCs (0.101 *versus* 0.017) and adenocarcinomas (0.112 *versus* 0.013) was 5.9- and 8.6-fold greater than that of their corresponding sporadic tumors ($P = 0.0002$ and $P = 0.0004$, respectively). Examples of various MA patterns for lung and breast cancers are presented in Fig. 1. Because artifacts resulting from PCR amplification may be mistaken for MAs, especially when minute amounts of input DNA are used, all examples of MAs were confirmed using DNA microdissected from replicate microsections. With the use of 20 polymorphic markers, at least one MA was present in 14 of 19 (74%) post-HD lung cancers *versus* 23 of 57 (40%) sporadic cases ($P = 0.017$), with the greatest differences between the two groups of lung carcinomas detected for the NSCLC, including adenocarcinomas. Although no differences were detected in the MA frequencies between dinucleotide or multinucleotide microsatellite repeat markers, the results varied considerably for individual markers. We did not find any of the microsatellite markers to be more sensitive than the others for detecting MA.

Both groups of breast carcinomas demonstrated a relatively low frequency of MAs, with the mean MA index of the post-HD breast tumors (mean, 0.021) about 4.2-fold greater than the mean index of sporadic cases (mean, 0.005; Fig. 2B). The difference, however, was not statistically significant ($P = 0.16$). In a combined analysis, post-HD lung and breast cancers had a significantly elevated MA index compared with sporadic cancers ($P = 0.001$). With the use of 20 polymorphic markers, at least one MA was present in 5 of 19 (26%) post-HD breast cancers *versus* 2 of 20 (10%) sporadic cases ($P = 0.24$; Table 4). In post-HD lung and breast tumors, there was no correlation between the MA index and the overall frequency of LOH and the age at which HD treatment was received, sex, HD stage, chest radiotherapy or chemotherapy, period of time between therapy and second cancer diagnosis, and the dose of radiotherapy received.

Minisatellite Mutations. Frameshift mutations were detected in all colon cancer cell lines used as controls. However, no mutations were detected in the minisatellite sequences at the *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH6*, and *hMSH3* genes examined in any of the lung and breast cancers arising in patients treated for HD.

Discussion

There is little information available regarding the genetic changes (12) involved in the pathogenesis of solid neoplasms that develop after treatment for HD. We analyzed the molecular profiles of 38 post-HD lung and breast carcinomas and compared them with those observed in sporadic lung ($n = 57$) and breast ($n = 20$) cancers. For patients who develop lung cancer after radiotherapy for HD, a positive interaction on a multiplicative scale has been detected between the carcinogenic effects of smoking and radiation (7), and the joint effects of smoking and radiation have been described previously in uranium miners exposed to radon (31) and in atomic bomb survivors (32). Breast carcinoma after HD radiotherapy is more likely to develop in women treated before age 30 years (10) and to present bilaterally and in medial quadrants in comparison with sporadic breast cancers (9, 33). The other clinicopathological characteristics of radiation-associated breast cancer after HD and sporadic tumors appear to be similar (33).

Our cases of post-HD lung and breast cancers did not demonstrate higher frequencies of *TP53* mutations than those observed in corresponding sporadic tumors (13, 34). Similar levels of *TP53* mutation have been noted in uranium miners

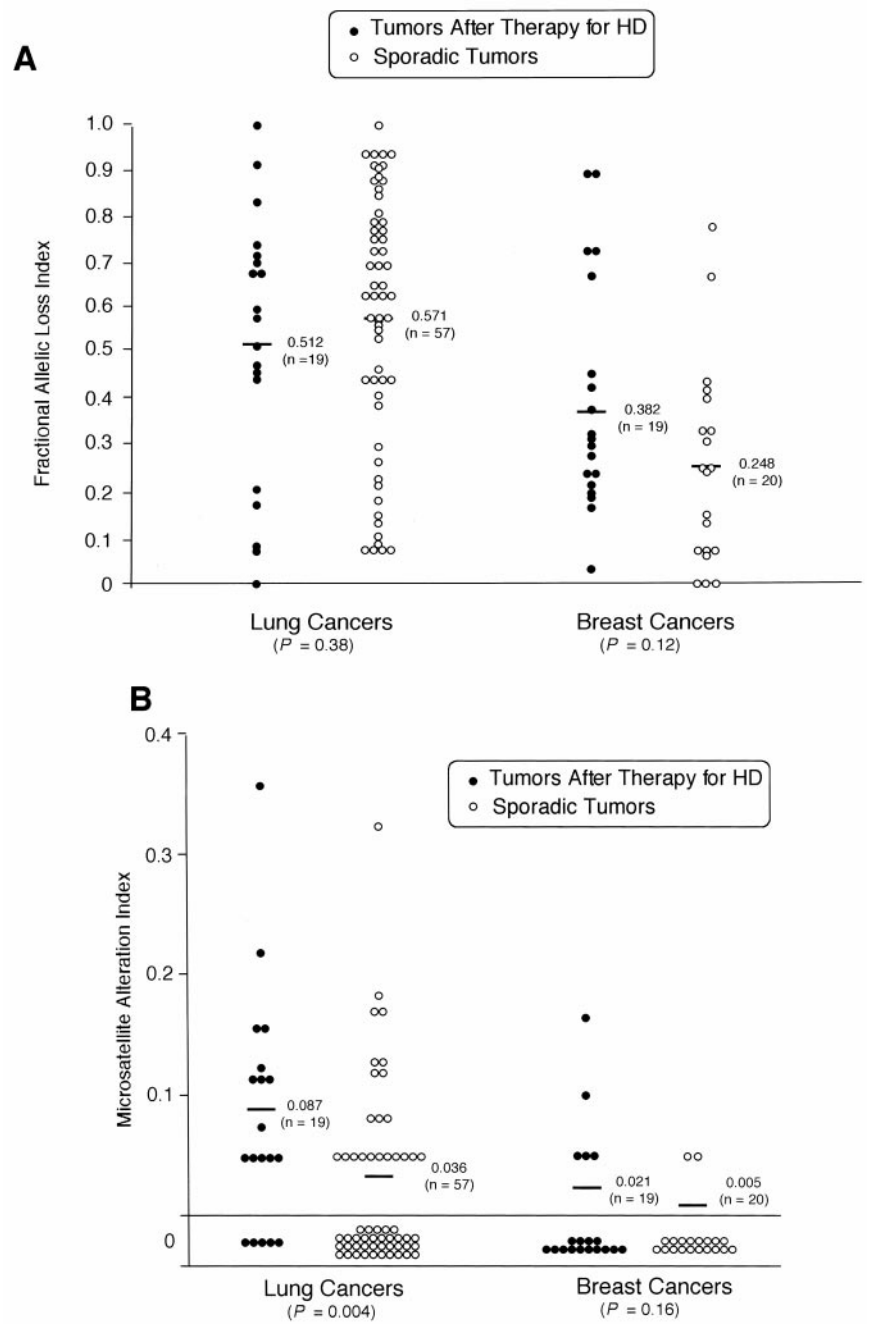

Fig. 2. Comparison of LOH and MA in post-HD disease lung and breast carcinomas and corresponding sporadic tumors. *A*, FAL index (an indicator of LOH at all chromosomal loci analyzed per case). *B*, MA index (an indicator of the total frequency of MAs per case). Although only post-HD lung carcinomas demonstrated a significantly increased frequency of MAs, the combined analysis of both breast and lung cancers revealed a highly significant elevation in the MA index ($P = 0.0013$) for the post-HD tumors.

exposed to radon and tobacco smoke (35) and in a small series of lung cancers after radiotherapy for HD (12). Although previous studies of *TP53* mutations in lung carcinomas after radiation exposure indicated a predominance of G:C > A:T transitions (12, 36), our post-HD lung tumors demonstrated predominantly transversions (70%). Although all patients with lung cancer after radiotherapy for HD in our survey were smokers, the pattern of *TP53* gene mutations resembled the spectrum of mutations described for the lung cancers of nonsmokers (37), with a predominance of G:C to C:G transversions. However, the small number of mutations in post-HD lung tumors ($n = 11$) dictates a cautious interpretation of this observation.

ras gene mutations occur in approximately 15–20% of NSCLCs, mainly in adenocarcinomas (20–30%; Ref. 13). Mutations in K-*ras* account for approximately 90% of *ras* mutations in lung adenocarcinomas, with 85% of these mutations affecting codon 12 (13). No increase in the frequency of K-*ras* gene mutations at codon 12 was detected in our cases of post-HD lung carcinoma. Similarly, no mutations at codons 12 and 13 of the K-*ras* gene were observed in lung cancers from uranium miners exposed to radon and tobacco smoke (35).

Many chromosomal deletions involving sites of known and putative TSGs have been described in clinically evident lung and breast cancers (13, 38). We determined the frequencies of LOH and MAs at several chromosomal regions in

Table 3 Comparison of LOH and MA frequencies between post-HD and sporadic lung cancers

Molecular abnormality	Post-HD tumors (n = 19)	Sporadic tumors (n = 57)	P
LOH			
3p22–24	12/17 (71%)	38/56 (68%)	1.00
3p21	14/19 (74%)	39/57 (68%)	0.78
3p14–21	6/10 (60%)	24/38 (63%)	1.00
3p14.2 (*FHIT* gene)	9/15 (60%)	38/52 (73%)	0.35
3p12	7/15 (47%)	31/46 (64%)	0.22
Any 3p	15/19 (79%)	53/57 (93%)	0.10
5q22 (*APC-MCC* region)	6/14 (43%)	16/43 (37%)	0.76
9p21	7/18 (39%)	29/49 (59%)	0.22
13q (*RB* gene)	4/13 (31%)	16/28 (57%)	0.18
17p (*TP53* gene)	13/14 (93%)	31/39 (80%)	0.41
Mean FAL index[a]	0.512	0.571	0.37
Mean FRL index	0.592	0.672	0.24
MA frequency	14/19 (74%)	23/57 (40%)	0.017
Mean MA index	0.087	0.036	0.004

[a] FAL index is an indicator of LOH at all microsatellite markers (n = 20) analyzed by case (range, 0–1); FRL index is an indicator of LOH at all chromosomal regions (n = 9) examined (range, 0–1); MA index is an indicator of LOH at all microsatellite markers (n = 20) analyzed by case (range, 0–1).

Table 4 Comparison of LOH and MA frequencies between post-HD and sporadic breast cancers

Molecular abnormality	Post-HD tumors (n = 19)	Sporadic tumors (n = 20)	P
LOH			
3p22–24	3/14 (21%)	5/12 (42%)	0.40
3p21	5/18 (28%)	7/17 (41%)	0.49
3p14–21	6/13 (46%)	3/13 (23%)	0.41
3p14.2 (*FHIT* gene)	9/17 (53%)	4/18 (22%)	0.086
Any 3p	11/19 (58%)	11/20 (55%)	1.00
5q22(*APC-MCC* region)	3/14 (21%)	3/14 (21%)	1.00
6q13–14	5/11 (45%)	0/14	0.01
6q22–27	4/15 (27%)	2/14 (14%)	0.65
Any 6q	8/19 (42%)	2/20 (10%)	0.03
8p	8/18 (44%)	5/20 (25%)	0.31
9p21	4/11 (37%)	0/15	0.02
11q	6/19 (32%)	7/20 (35%)	1.00
13q (*RB* gene)	6/16 (38%)	5/19 (26%)	0.72
17p (*TP53* gene)	16/19 (84%)	7/15 (47%)	0.03
17q (*BRCA1* region)	7/16 (44%)	5/18 (28%)	0.48
FAL index mean[a]	0.382	0.248	0.12
FRL index mean	0.429	0.299	0.17
MA frequency	5/19 (26%)	2/20 (10%)	0.24
MA index mean	0.021	0.005	0.16

[a] FAL index is an indicator of LOH at all microsatellite markers (n = 20) analyzed by case (range, 0–1); FRL index is an indicator of LOH at all chromosomal regions examined (n = 13); MA index is an indicator of alterations at all microsatellite markers (n = 20) analyzed by case (range, 0–1).

post-HD lung and breast tumors and in sporadic carcinomas of these types. Of interest, the overall frequency of LOH at all regions was similar in the two groups of lung and breast cancers. For individual regions, the only significant differences were noted at the 6q13–14, 9p 21, and *TP53* loci in breast carcinomas, in which post-HD tumors demonstrated a significantly higher frequency of allelic loss. Because of the small sample size and numerous comparisons, we feel that cautious interpretation of the data is advisable until additional data are available.

A significantly greater frequency of MAs was present in the post-HD lung tumors of all histological types. Although a higher frequency of MAs (4.2-fold) was present in post-HD breast carcinomas than in sporadic cases, these differences were not significant. However, the overall frequency of MAs in breast tumors was lower than that in lung cancers. The differences between the lung and breast tumors may be related to organ-specific differences in radiation sensitivity or may reflect the compounding effects of exposure to smoking-related carcinogens. Chronic smokers have an increased incidence of MAs in the bronchial epithelium (30).

MAs have been reported in a variety of sporadic cancers, including those of the lung and breast. Although a link between MAs and DNA repair mechanisms has not been proven, MAs likely constitute evidence for some type of genomic instability (39). Because most microsatellite sequences arise in noncoding regions of the genome, they are not in the direct pathway of carcinogenesis. Whereas lengthy microsatellite sequences are uncommon in coding regions, smaller repeats (minisatellites) are occasionally present. In hereditary nonpolyposis colon cancer patients (who have defects in their DNA repair enzymes), size changes affecting microsatellites and minisatellites are present at many noncoding regions and some coding regions, including those of known or putative oncogenes. Because alterations in coding sequences may result in frameshift mutations (40, 41), it is possible that MAs also could result in the inactivation of critical key genes in cancer pathogenesis. However, changes in the minisatellite sequences at the *TGF-βRII*, *IGFIIR*, *BAX*, *hMSH3*, and *hMSH6* genes were not detected in our post-HD cancers. Our results suggest that mutations in DNA repair enzymes are unlikely to be the primary mechanism involved in the increased frequency of MAs in post-HD lung and breast tumors. MAs occurring in polymorphic markers in noncoding regions of the genome may be repaired less efficiently than those occurring in the coding sequences of crucial genes.

Mechanisms by which elevated rates of MA occur in post-HD cancers are not clear. Evidence exists for increased frequencies of genomic instability after radiation exposure in humans (42, 43), cell cultures (44), and experimental animals (45). An increased frequency of microsatellite instability has been detected previously in therapy-related leukemia and pediatric secondary malignant neoplasms (46, 47). In addition, increased genomic instability at several chromosomal loci has been observed in human radiation-associated thyroid carcinomas that developed after the Chernobyl accident (48, 49). In cell culture systems, ionizing radiation induces widespread genomic instability that is dose-dependent (44, 50). However, in our post-HD lung and breast tumors, there was no correlation between the MA index and chest radiotherapy or dose of radiotherapy received. Although several of the cytotoxic drugs used to treat HD, including mechlorethamine and procarbazine, cause pulmonary tumors in laboratory animals (51), evidence for the possible contribution of chemotherapy to subsequent lung cancer excesses after HD is conflicting (2, 6). Whether cytotoxic drugs might play a role in the development of excess genomic instability is not known, and we found no difference in MA rates between patients who received chemotherapy plus radiotherapy (n = 16) and those receiving radiotherapy alone (n = 22). A relatively high frequency of MAs has been described in lung cancers arising in young nonsmoking subjects (52), suggesting a genetic predisposition. The ages of our patients with post-HD lung and breast cancers were significantly lower than those of patients with sporadic tumors. MAs have also been described previously in HIV-associated tumors, including those of the lung, and may be involved in their

pathogenesis (26, 53, 54). Other immunosuppressive states may also result in minisatellite instability (55). It is noteworthy that defects in immunological function are present at diagnosis of HD and persist for long periods after completion of therapy (56).

Our findings suggest that widespread genomic instability, as manifested by the development of increased numbers of MAs, occurs frequently in lung and breast tumors after radiation therapy for HD. Although the mechanism underlying the development of increased MAs is unknown, future research is warranted to address the possible contribution of these alterations to the pathogenesis of selected second neoplasms after treatment for HD. Our results also underscore the importance of additional epidemiological and laboratory studies to clarify the relationship between tobacco, immunological factors, radiation, chemotherapy, and other possible influences in the development of second cancers after HD.

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